

REMARKS

Summary of Invention

The invention features *lin-37* nucleic acids encoding polypeptides capable of inhibiting cellular proliferation and vectors and host cells containing these nucleic acids.

The Office Action

Claims 1, 4-7, 10-18, 25, 34, 36, 38-40, 42, 44-46, 48, 50-52, 54, 56-58, 60, 61, 63, and 64 are pending and considered in the present Office Action. Claims 1, 4-7, 10-18, 25, 34, 36, 38-40, 42, 44-46, 48, 50-52, 54, 56-58, 60, 61, 63, and 64 are rejected under 35 U.S.C. § 101 and § 112, first paragraph. Each of these rejections is addressed as follows.

Claim Amendments

Claims 1, 10, 16, 18, 25, 34, 40, 46, 58, and 61 have been amended to recite “at least 95%” sequence identity. Claims 36, 42, 48, 54, and 60 have been amended to recite “99% or greater” sequence identity. Support for these amendments is found at page 10, lines 21-23 and page 36, lines 22-24. Claims 16-18, 46, 48, 50-52, 54, 56, and 57 have been amended to recite “isolated cells that contain a substantially pure nucleic acid encoding a *lin-37* polypeptide.” Support for these amendments is found on page 24, lines 4 to page 25, line 20.

Rejections under 35 U.S.C. § 101 and § 112, first paragraph

Claims 1, 4-7, 10-18, 25, 34, 36, 38-40, 42, 44-46, 48, 50-52, 54, 56-58, 60, 61, 63, and 64 are rejected under 35 U.S.C. § 101 and § 112, first paragraph. The Examiner maintains that the claimed invention lacks a specific, substantial asserted utility or a well-established utility that would enable one skilled in the art to use the invention. Applicants maintain their position that *lin-37* nucleic acids and polypeptides are useful for the modulation of cell proliferation, for identifying homologs in other species, and for identifying candidate compounds that modulate synMuv expression or cell death activity and that such uses constitute a specific and substantial utility. Thus, the rejection remains respectfully traversed.

The Federal Circuit in *In re Brana*, 51 F.3d 1560 (Fed. Cir. 1995) has articulated the standard to be applied by the PTO in any challenge to an assertion of utility. In this case, the Court stated (at page 1566):

the PTO has the initial burden of challenging a presumptively correct assertion of utility in the disclosure. [citation omitted]. Only after the PTO provides evidence showing that one of ordinary skill in the art would reasonably doubt the asserted utility does the burden shift to the applicant to provide rebuttal evidence sufficient to convince such a person of the invention's asserted utility.

Applicants, therefore, submit that it is the Examiner who bears the burden of challenging a presumptively correct assertion of utility. As is discussed below, the Examiner has failed to provide the necessary evidence required to meet this burden.

Applicants have clearly disclosed a number of specific utilities for their claimed invention. *lin-37* functions in the *C. elegans* synMuv pathway, which is a conserved tumor suppressor pathway also involved in cancer pathogenesis in humans. Applicants disclose that synMuv genes in *C. elegans*, such as *lin-37*, “are part of a pathway which may be used as a genetic and biochemical model system for tumor suppression and, conversely, cancer” (page 3, first paragraph). Applicants state that “genetic and molecular analyses of Muv and Vul animals have defined a Ras signal transduction pathway that mediates induction of the hermaphrodite vulva” (page 2, first paragraph), and that “Ras pathways have been found to control cell proliferation in a range of organisms from the yeast *S. cerevisiae* to humans” (page 2, first paragraph). Similar to the retinoblastoma (Rb) protein, a tumor suppressor that negatively regulates Ras and is found to be mutated in eye cancers, the synMuv genes act as negative regulators of the signaling pathway. In fact, the sequences of several synMuv genes (e.g. *lin-35*, *lin-53*, and *lin-55*) have homology to proteins in the Rb tumor suppressor pathway family of proteins. Accordingly, Applicants disclose that synMuv pathway genes and proteins may be used to identify genes that are part of the mammalian pathway and to identify genes, proteins, and therapeutic compounds which modulate this pathway. The Examiner submits that the present specification states that *lin-37* does not have homology to known tumor suppressor genes (present Office Action, page 3, lines 10-11). Such an observation is insufficient to support a rejection for lack of utility, as it does not necessitate the

conclusion that no homolog or ortholog exists. As evidence of this, Applicants submit that a *lin-37* homolog in the fruitfly *Drosophila melanogaster* was recently found to function in a retinoblastoma tumor suppressor protein (pRb) complex (Korenjak et al., *Cell* 119:181-193 (2004), p. 182, column 2, paragraph one and p. 185, column 2, paragraphs 4-5; reference enclosed as Appendix A). Clearly, taken together with the highly conserved function of Rb and Ras, this data supports Applicants' earlier assertion that synMuv proteins, including *lin-37*, function to antagonize Ras in mammals. Because Applicants disclose specific utilities for *lin-37* associated with this function, and this disclosure has not been rebutted by evidence, the utility rejection should be withdrawn.

In evaluating an assertion of credible utility, Applicants further note that the Revised Interim Utility Guidelines Training Materials require:

where an applicant has specifically asserted that an invention has a particular utility, that assertion cannot simply be dismissed by Office personnel as being "wrong". Rather, Office personnel must determine if the assertion of utility is credible (i.e., whether the assertion of utility is believable to a person of ordinary skill in the art based on the totality of evidence and reasoning provided). An assertion is credible unless (A) the logic underlying the assertion is seriously flawed, or (B) the facts upon which the assertion is based are inconsistent with the logic underlying the assertion. (page 5, paragraph 1; emphasis added)

Applicants have disclosed the use of *lin-37* nucleic acids and polypeptides for the modulation of cell proliferation, for identifying homologs in other species, and for identifying candidate compounds that modulate synMuv expression or cell death activity.

The synMuvs act to negatively regulate vulval induction. At a molecular level, vulval induction is controlled by a Ras pathway. Ras pathway members have been shown to mutate in a variety of human cancers to give an increased level of pathway signalling. Applicants have shown that a pathway related to the tumor suppressor Rb negatively regulates Ras pathway signalling. Applicants have also identified a number of synMuv genes which act as part of this Rb-related pathway. The striking parallel between the Rb pathway in mammals and the Rb-related pathway in nematodes indicates that the logic underlying Applicants' assertion of utility cannot be "seriously flawed". The finding that a *lin-37* homolog functions in an Rb-related complex in fruitflies provides further evidence that Applicants have disclosed a credible utility for *lin-37* nucleic acids and polypeptides.

The Examiner has failed to carry the burden, as articulated in *Brana* (see above), to challenge Applicants' assertion of utility. No evidence has been presented explaining why the association of *lin-37* with cell proliferation and cancer is contrary to scientific reasoning. The Examiner purports that "[t]he specification has not identified a Ras pathway in mammals which would be orthologous to the Ras pathway of the nematode, nor does the specification teach how the commitment of cells to vulval induction in the nematode has a nexus to a biochemical signaling pathway in mammals that would be causative, rather than associative, with the cancerous phenotype in mammals" (present Office Action, page 3, lines 13-17). Applicants, however, assert that extensive evidence

exists indicating that the synMuv pathway has a credible functional relationship to the Ras pathway in mammals. Mutations in Ras are found in a variety of human cancers. Similar to the function of Rb in other organisms, *lin-37* antagonizes Ras in *C. elegans*. Recent evidence demonstrates that *lin-37* homologs function in an Rb complex in fruitflies. These data do not contradict, but rather support, the highly conserved nature of Ras and Rb in various organisms, including mammals. Accordingly, the related rejections under 35 U.S.C. § 101 and § 112, first paragraph should be withdrawn.

Claims 1, 4-7, 10-15, 25, 34, 36, 38-40, 42, 44, 45, 58, 60, 61, 63, and 64 stand rejected under 35 U.S.C. § 112, first paragraph for lack of enablement because the specification, while being enabling for nucleic acids encoding SEQ ID NO:1, and isolated cells comprising SEQ ID NO:1, does not reasonably provide enablement for nucleic acids encoding a polypeptide having at least 85% sequence identity to SEQ ID NO:1.

While Applicants do not agree with the basis for the Examiner's rejection, in the interest of obtaining allowance, they have amended the claims to nucleotide and amino acid sequences having "at least 95%" sequence identity to SEQ ID NO: 1. Applicants submit that these claim amendments render the rejection under 35 U.S.C. § 112, first paragraph moot, and may now be withdrawn.

Claims 16-18, 46, 48, 50-52, 54, 56, and 57 stand rejected under 35 U.S.C. § 112, first paragraph for lack of enablement. The Examiner states that the specification is not enabling for gene therapy.

While Applicants do not agree with the basis for the Examiner's rejection, in the interest of obtaining allowance, they have amended the claims to render this rejection moot. Applicants submit that the limitation of the amended claims to "isolated cells" specifies that the cells are in vitro.

Claims 16-18, 46, 48, 50-52, 54, 56, and 57 have been amended to recite "isolated" cells that contain a substantially pure nucleic acid encoding a *lin-37* polypeptide. Claim 17 has been cancelled. Applicants submit that this rejection under 35 U.S.C. § 112, first paragraph now be withdrawn.

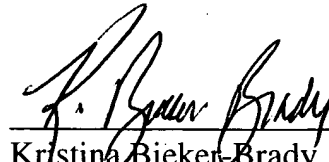
CONCLUSION

Applicants submit that the claims are now in condition for allowance, and such action is respectfully requested. Should the Examiner continue to maintain the rejections under 35 U.S.C. § 101 and § 112, first paragraph, Applicants request a teleconference between Kristina Bieker-Brady, Ph.D., P.C., the attorney of record in the present application, the Examiner, and the Examiner's Supervisor, Christina Chan. Dr. Bieker-Brady can be reached at (617) 428-0200. Enclosed are a petition to extend the period for replying for three months, to and including November 22, 2004, a check for \$490.00 for

the required petition fee. If there are any additional charges or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Date: November 22, 2004



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Native E2F/RBF Complexes Contain Myb-Interacting Proteins and Repress Transcription of Developmentally Controlled E2F Target Genes

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Summary

The retinoblastoma tumor suppressor protein (pRb) regulates gene transcription by binding E2F transcription factors. pRb can recruit several repressor complexes to E2F bound promoters; however, native pRb repressor complexes have not been isolated. We have purified E2F/RBF repressor complexes from *Drosophila* embryo extracts and characterized their roles in E2F regulation. These complexes contain RBF, E2F, and Myb-interacting proteins that have previously been shown to control developmentally regulated patterns of DNA replication in follicle cells. The complexes localize to transcriptionally silent sites on polytene chromosomes and mediate stable repression of a specific set of E2F targets that have sex- and differentiation-specific expression patterns. Strikingly, seven of eight complex subunits are structurally and functionally related to *C. elegans* synMuv class B genes, which cooperate to control vulval differentiation in the worm. These results reveal an extensive evolutionary conservation of specific pRb repressor complexes that physically combine subunits with established roles in the regulation of transcription, DNA replication, and chromatin structure.

Introduction

The retinoblastoma susceptibility gene product (pRb) is inactivated in a variety of tumors (Weinberg, 1995). pRb and the pRb-related proteins p107 and p130 constitute the “pocket” protein family, named for a shared viral oncoprotein binding domain. Pocket proteins cooperate with heterodimeric E2F transcription factors to regulate

cell growth and differentiation and provide a control mechanism that is present in both plants and animals (Frolov and Dyson, 2004; Shen, 2002).

The roles of pRb and E2F proteins in the coordination of gene transcription with cell cycle progression have been studied extensively (Cam and Dynlacht, 2003; DeGregori, 2002; Trimarchi and Lees, 2002): in G₀ and early G₁ phases of the cell cycle, E2F repressor complexes prevent expression of a diverse set of genes that encode cell cycle regulators and products essential for DNA synthesis. Transcriptional repression is affected by the pocket proteins that bind to a conserved C-terminal domain in E2F proteins. As cells progress through G₁, activator E2Fs override the effects of repressor E2F complexes and generate a controlled pulse of gene expression. In mammalian cells, E2F4, E2F5, and E2F6 are thought to function primarily as repressors, whereas E2F1, E2F2, and E2F3a are thought to drive gene expression. In *Drosophila*, the analogous functions are provided by dE2F1, a potent activator of transcription, and dE2F2, a dedicated repressor (Frolov et al., 2001). In both systems, the induction of E2F-dependent transcription is driven by cyclin-dependent kinases and is controlled, at least in part, by the phosphorylation of pRb-related proteins.

pRb and E2F family members play important roles during development, particularly in settings where cell division and differentiation need to be tightly coordinated. For example, mice lacking pRb or combinations of pRb and p107 or p130 have tissue-specific developmental defects that result in embryonic lethality (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992, 1996). Genetic studies also reveal specific roles for individual E2F family members. Interestingly, several E2F mutations give tissue-specific abnormalities that do not seem to result from defects in cell proliferation (Cloud et al., 2002; Lindeman et al., 1998; Storre et al., 2002). dE2F2 mutant flies have fertility defects, and dE2F2 and dDP are required for several key events during oogenesis, including cessation of DNA synthesis and chorion gene amplification in follicle cells (Cayirlioglu et al., 2001; Stevaux and Dyson, 2002). In *C. elegans*, mutation of E2F or DP genes (*eff-1* and *dpl-1*) has only a limited effect on cell proliferation (Ceol and Horvitz, 2001). Instead, mutant alleles of *eff-1* and *dpl-1* are members of the synthetic *multivulva* (synMuv) class B that, when combined with mutations in synMuv class A or synMuv class C genes, induce an abnormally high number of precursor cells to adopt a vulval cell fate, resulting in animals with multiple vulvae (Ceol and Horvitz, 2001, 2004). *lin-35*, encoding the only pRb family member of *C. elegans*, is also a synMuv class B gene, and it has been proposed that the change in cell fate that underlies the synMuv phenotype may be caused in part by the loss of an E2F/pRb repressor complex (Ceol and Horvitz, 2001; Lu and Horvitz, 1998).

The idea that E2F proteins have tissue-specific, developmentally regulated functions is supported by the identification of novel E2F regulated genes in human, mouse, and fly. In addition to cell cycle-related E2F targets,

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these studies revealed numerous genes that have developmental functions or display a strictly tissue-specific expression pattern (DeGregori, 2002). Analysis of the E2F transcriptional program in *Drosophila* indicates that there are at least two different types of E2F regulation (Dimova et al., 2003): expression of cell cycle-regulated E2F targets is primarily dependent on dE2F1/dDP-mediated activation and is repressed by RBF1 (A group genes). In contrast, other E2F targets are actively repressed in proliferating cells by dE2F2, dDP, and either RBF1 or RBF2, and these genes are expressed in developmentally regulated patterns (E group genes). These two types of regulation appear to be combined in differing proportions over the spectrum of E2F targets, generating a broad variety of E2F control.

Pocket proteins repress transcription in several different ways. They can counteract E2F-mediated transactivation simply by binding to and masking the E2F activation domain (Frolov and Dyson, 2004). In addition, they actively repress transcription by recruiting corepressor complexes to E2F. Several of the complexes that have been linked to pRb have enzymatic activities directed toward chromatin or recognize particular histone modifications, suggesting that they impinge on chromatin structure (Schneider et al., 2002; Zhang and Dean, 2001).

Currently, more than 120 proteins have been reported to associate with pRb, and a wide assortment of chromatin-modifying and binding complexes have been implicated in pRb-mediated repression (Frolov and Dyson, 2004). Many pRb binding proteins have been studied using *in vitro* binding and coimmunoprecipitation assays, often following forced overexpression of one or both putative interaction partners. It is unclear which of the many reported interactions are physiologically relevant. The plethora of proposed corepressors raises a series of critical questions. Which interactors are really required for pRb to repress transcription? Under which circumstances and on which genes do these complexes act? Since pRb and E2F family members regulate genes that are required for a broad range of cellular functions, it is possible that different corepressors are utilized at different sets of target genes. It is also conceivable that pocket proteins recruit different repressors to the same targets in different cellular conditions (Ait-Si-Ali et al., 2004; Narita et al., 2003).

Paradoxically, given the extensive literature on pRb-associated proteins, all attempts to purify native pRb repressor complexes have been unsuccessful. Chromatographic fractionation of E2F complexes from mammalian cells is complicated by the fact that they constitute an aggregate collection of many different combinations of E2F/DP/pRb family members. Moreover, purification of endogenous protein complexes from mammalian cells traditionally relies on the use of rapidly dividing cell lines, such as HeLa, which allow accumulation of sufficient starting material. It is unclear whether tumor cell lines are a good source for pRb repressor complexes, as pRb is often inactivated in these cells.

In this study, we have taken advantage of the relative simplicity of the *Drosophila* dE2F/RBF network to isolate two related, native multisubunit complexes containing RBF, dE2F2, and dMyb-interacting proteins (dREAM). In agreement with a role in transcriptional repression, these complexes localize to nontranscribed sites on

polytene chromosomes. Depletion of specific subunits by RNAi has identified a set of sex- and differentiation-specific dE2F target genes that are regulated by these complexes *in vivo*. Strikingly, seven dREAM subunits are related to *C. elegans* synMuv class B genes. The existence of putative homologs of dREAM subunits in mammals and the ability of pocket proteins to interact with these suggests a remarkable degree of evolutionary conservation in the mechanism of pocket protein action.

Results

Chromatographic Separation of RBF Complexes

The *Drosophila* genome encodes two pocket proteins, RBF1 and RBF2, and two E2F proteins, dE2F1 and dE2F2, that act in heterodimers with a common partner, dDP. We reasoned that this streamlined version of the E2F/pRb network would greatly simplify the chromatographic separation of native complexes. We subjected *Drosophila* embryo nuclear extracts to gel filtration to verify the presence of RBF complexes (Figure 1A). RBF1 was detected by Western blot in many fractions ranging in apparent molecular weight from 66 kDa to 1.2 MDa. RBF2 was detected in a narrower peak with an apparent molecular weight of 669 kDa to 1.2 MDa. dDP was likewise detected in fractions ranging in molecular weight from 443 kDa to 1.2 MDa. These findings suggest that *Drosophila* embryos contain multisubunit dE2F/RBF complexes.

Next, we subjected extracts to ion exchange chromatography. This resolved three peaks of RBF1 activity (Figure 1B). Peak I contained RBF1 but did not contain RBF2, dE2F, or dDP. When peak I was subjected to gel filtration, RBF1 eluted with an apparent molecular weight of 100 kDa, close to its theoretical molecular weight (91.8 kDa), suggesting that peak I contains monomeric RBF1 (data not shown). RBF1, dE2F1, and dDP coeluted in peak II. During subsequent gel filtration, these three proteins coeluted with an estimated molecular weight of 500 kDa (data not shown). Analysis of peak III revealed the presence of RBF1, RBF2, dE2F2, and dDP. These four proteins coeluted during gel filtration with an apparent molecular weight of 669 kDa to 1.2 MDa (data not shown).

Previous studies have shown that RBF1 associates with both dE2F1 and dE2F2, whereas RBF2 interacts exclusively with dE2F2, and these binding specificities are reflected in our elution profile (Stevaux et al., 2002). Interestingly, peak III fractions contain both RBF1 and RBF2 even though they do not interact with each other ([Stevaux et al., 2002] see also Figure 2D). This suggests that peak III contains two separate dE2F2/RBF1 and dE2F2/RBF2 complexes with similar subunit composition. The molecular weight of these complexes (669 kDa to 1.2 MDa) indicates that they contain additional subunits. We therefore purified dE2F2/RBF complexes present in peak III.

Purification of dE2F2/RBF Complexes

dE2F2/RBF complexes were purified by classical chromatography (Figure 2A). dE2F2, dDP, RBF1, and RBF2 coeluted from the final gel filtration column with an apparent molecular weight of 669 kDa to 1.2 MDa (Figure



Figure 1. Native RBF Complexes in *Drosophila*

(A) Nuclear extracts were subjected to Superose 6 gel filtration. Fractions were analyzed by Western blot using specific antibodies as indicated. Fraction numbers are denoted on top, size standards on the bottom. IN, input.

(B) Nuclear extracts were fractionated over Q Sepharose. Fractions were analyzed by Western blot using specific antibodies as shown. Three RBF1 peaks are indicated on top. IN, input; FT, flowthrough.

2C). Silver staining detected seven bands that perfectly coeluted with the Western signals (Figure 2B). These polypeptides were present in similar stoichiometric amounts, with the exception of one (55 kDa) that was stained more intensely. Peptide mass fingerprinting revealed that the 55 kDa band comprised two distinct polypeptides (see below).

Identification of copurifying polypeptides revealed Twilight (also known as Mip130; from here on referred to as Mip130/TWIT), RBF1, RBF2, dMyb, dDP, dE2F2, CAF1p55, and Mip40 (Figure 2B). The identity of these polypeptides was confirmed by Western blot (Figure 2C). Intriguingly, Mip130/TWIT, dMyb, CAF1p55, and Mip40 have recently been identified as components of a dMyb complex that regulates chorion gene amplification in follicle cells (Beall et al., 2002). The fifth subunit of the dMyb complex, Mip120, was apparently absent from our final preparation as judged by silver staining (Figure 2B). However, Western analysis with Mip120-specific antibody demonstrated that Mip120 coeluted with other complex subunits throughout the fractionation. The Mip120 signal became progressively weaker during purification but was still detectable in fractions eluting from the final gel filtration column (Figure 2C and data not shown), suggesting that Mip120 might have been progressively lost or degraded. Indeed, several results presented below suggest that Mip120 is a bona fide complex subunit. Since these complexes are a composite of known transcriptional regulators, we refer to them by the acronym dREAM (*Drosophila* RBF, E2F, and Myb-interacting proteins).

To verify physical association between dREAM subunits, we subjected Q Sepharose peak III to immunoprecipitation with RBF1- and RBF2-specific antibodies in the presence of ethidium bromide to minimize DNA-mediated interactions (Figure 2D). Control antibodies (α -Myc) did not precipitate any of the proteins tested. Both RBF1- and RBF2-specific antibodies precipitated Mip130/TWIT, dDP, CAF1p55, dE2F2, and Mip40. Mip120 was precipitated by RBF1-specific antibodies, but only a faint Mip120 signal was evident in the RBF2 precipitate. In agreement with previous studies, RBF1-specific antibodies failed to precipitate RBF2 and vice versa, indicating the presence of two distinct dREAM complexes.

The *Drosophila* E2F transcription program has previously been dissected in S2 cells by RNAi. To determine whether S2 cells contain dREAM complexes, we generated stable lines that express Flag-tagged versions of RBF1 and RBF2. α -Flag antibodies were used to precipitate RBF1 and RBF2, and associated proteins were detected by Western blot (Figure 2E). As expected, dE2F1, dE2F2, and dDP coprecipitated with RBF1, while only dE2F2 and dDP coprecipitated with RBF2. Abundant chromatin-associated proteins, such as histone H3 and HP1, were not coprecipitated, demonstrating the specificity of the assay. Mip130/TWIT, dMyb, CAF1p55, and Mip120 were each coprecipitated with both RBF1 and RBF2.

In summary, our biochemical studies define two native dREAM complexes present in both embryos and S2 cells that contain Mip130/TWIT, Mip120, dMyb, CAF1p55, dE2F2, dDP, and Mip40 in addition to either RBF1 or RBF2.

dREAM Complexes Bind Repressed Chromatin

As a first step toward understanding the *in vivo* role of these complexes, we stained polytene chromosomes with antibodies directed against dREAM subunits (Figure 3). dE2F2, RBF1, Mip120, and Mip130/TWIT antibodies each highlighted numerous bands. Coimmunostaining revealed extensive colocalization between these subunits (Figures 3A–3D). These results support the biochemical evidence that Mip130/TWIT and Mip120 associate with dE2F2 and RBF1 and suggest that these proteins function in concert with one another at many discrete chromosomal locations.

Next, we sought to determine if the sites occupied by dREAM subunits represent active or inactive chromatin. We made use of an antibody directed against phosphorylated RNA polymerase II (pol II H5) that yields numerous bands corresponding to actively transcribed regions of the genome (Figure 4A). Despite the fact that both Mip130/TWIT and pol II antibodies each stain hundreds of bands, there is, strikingly, no overlap between these two patterns (Figure 4B). This indicates that dREAM complexes primarily associate with transcriptionally silent regions of the genome, consistent with a role in transcriptional repression.

An antibody recognizing Polycomb (Pc), a well-char-

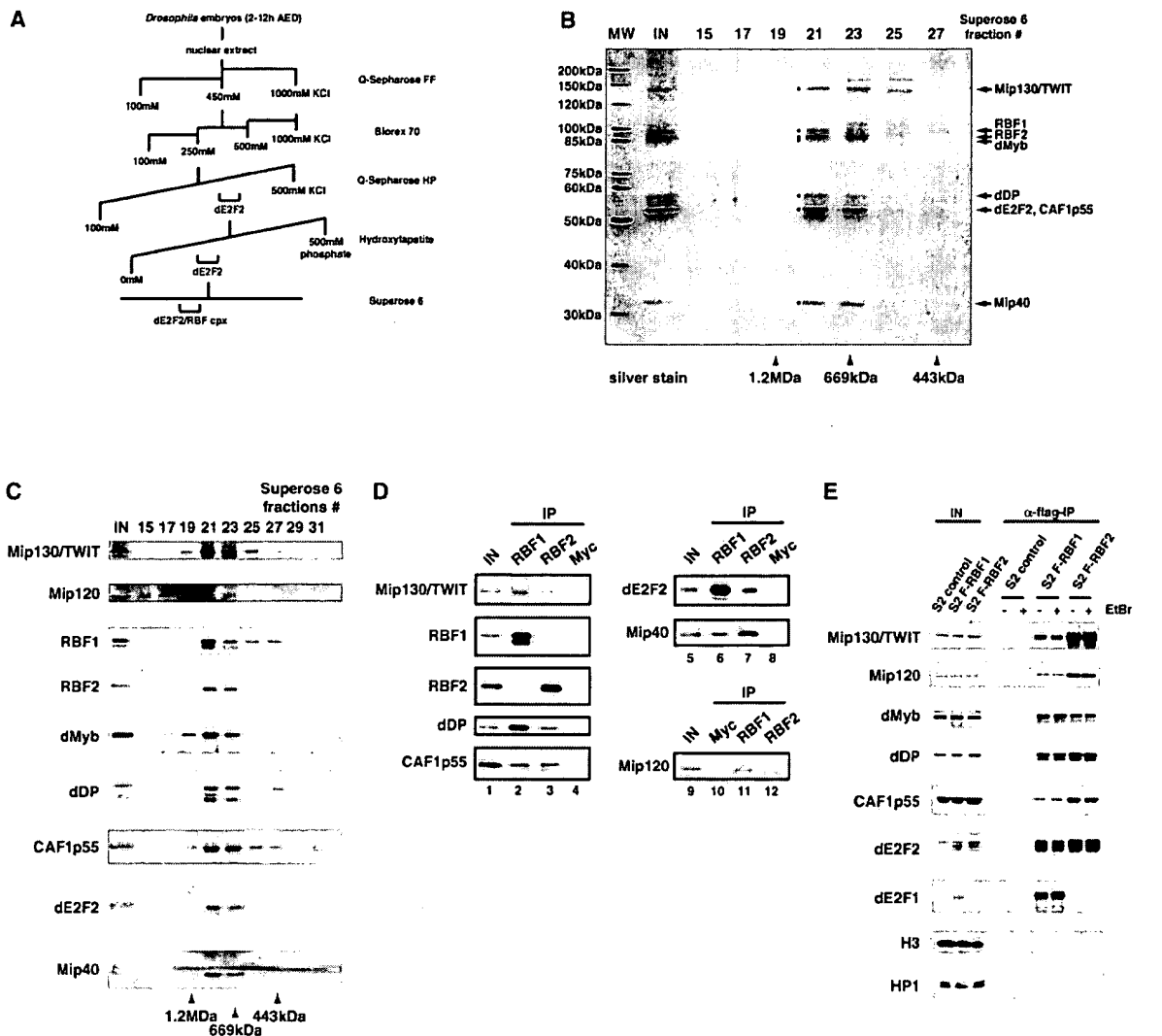


Figure 2. Purification of dREAM Complexes

(A) Schematic representation of procedure.

(B) Fractions eluting from the final column were visualized by silver staining. Polypeptides coeluting with RBF1, RBF2, dE2F2, and dDP Western signals are marked with solid black circles. SDS PAGE and gel filtration size markers are shown on the left and on the bottom, respectively. dREAM subunits are indicated by arrows. MW, molecular weight markers; IN, input.

(C) Fractions shown in (B) were analyzed by Western blot using specific antibodies as indicated.

(D) Fractions derived from Q Sepharose peak III (see Figure 1B) were precipitated with antibodies directed against RBF1, RBF2 and c-Myc (control) in the presence of 50 μ g/ml ethidium bromide, as indicated on top. Immunoprecipitates were subjected to Western blot using specific antibodies as indicated on the left. IN, input.

(E) Extracts derived from an S2 line (S2 control) and from S2 lines stably expressing Flag-tagged RBF1 (S2 F-RBF1) and RBF2 (S2 F-RBF2) were precipitated with α -Flag antibodies as shown on top. Immunoprecipitates were subjected to Western blot using specific antibodies as indicated. Precipitations were carried out in absence or presence of 200 μ g/ml ethidium bromide (EtBr) as indicated. IN, input; α -Flag-IP, α -Flag immunoprecipitates.

acterized repressor that functions to maintain stable repression of its targets, also stains a set of bands that do not overlap with transcriptionally active chromatin (Figure 4A). However, costaining with Mip130/TWIT and Pc antibodies revealed that Pc sites are not bound by Mip130/TWIT and vice versa (Figure 4C). Thus, dREAM complexes function at sites that are distinct from those targeted by Pc.

dREAM Complexes Bind to Histone H4 Tails

Nontranscribed chromatin is characterized by low levels of histone acetylation. pRb has been proposed to re-

press transcription by assembling complexes with chromatin-directed enzymatic activities such as histone deacetylases. dREAM complexes appear to lack chromatin-modifying enzymes. Notably, dRPD3, the *Drosophila* ortholog of the human Rb-associated HDAC1 histone deacetylases, is not a stoichiometric component of dREAM.

Since many repressors interact directly with histones, we examined the histone binding properties of dREAM complexes, and, given their localization to nontranscribed regions, we asked whether binding was influenced by histone acetylation. Fractions enriched for

dREAM complexes (peak III, see Figure 1B) were incubated with immobilized histone H4 tail peptides. As controls, we tested fractions containing monomeric RBF1 (peak I) or dE2F1/RBF1 complex (peak II). We compared binding of RBF1, RBF2, and Mip130/TWIT to nonacetylated and tetraacetylated histone H4 tails. Monomeric RBF1 bound weakly to the acetylated H4 tail but did not bind the nonacetylated peptide (Figure 4D, lanes 2 and 3). Likewise, RBF1 present in the dE2F1/RBF1 complex failed to interact with either H4 peptide (lanes 6 and 7). By contrast, the RBF1, RBF2, and Mip130/TWIT dREAM subunits clearly bound to nonacetylated H4 tail (lane 10). Strikingly, this interaction was not detected when the H4 tail was acetylated (lane 11). We conclude that dREAM complexes bind histone H4 tails *in vitro* and that this interaction is abrogated by acetylation.

Targets of dREAM Complexes

Recently, we have identified dE2F-regulated genes by a combined RNAi/microarray approach (Dimova et al., 2003). We depleted dREAM subunits by treating S2 cells with RNAi and monitored changes in transcription of known dE2F targets by Northern blot to identify genes regulated by dREAM (Figures 5A and 5B). RNAi treatments did not result in changes of cell cycle profiles or BrdU incorporation patterns (Dimova et al., 2003; Frolov et al., 2003; see Supplemental Figure S1 at <http://www.cell.com/cgi/content/full/119/2/181/DC1/>). We concentrated on two classes of dE2F-regulated genes: cell cycle-regulated A group genes and E group genes, which are permanently repressed in S2 cells and display a sex- or tissue-specific expression pattern (Dimova et al., 2003). In agreement with our previous findings, depletion of RBF1 upregulated A group genes but had no effect on the transcription of E group genes, which are repressed by RBF1 and RBF2 in a redundant fashion (Figure 5A, compare lanes 1 and 3; Dimova et al., 2003). As expected, dE2F2 depletion did not change A group gene expression but led to a massive increase of E group gene transcription (compare lanes 1 and 4). Strikingly, depletion of Mip130/TWIT and Mip120 also resulted in a robust deregulation of E group transcription but had no effect on A group gene transcription. Thus, Mip130/TWIT, Mip120, and dE2F2 are required to silence E group genes in S2 cells (compare lanes 1, 2, 5, and 6).

Depletion of Mip120 had no significant effect on dE2F2 protein levels, but, curiously, depletion of dE2F2 reduced the Mip130/TWIT levels and vice versa (Figure 5B). Similarly, depletion of dDP results in the simultaneous reduction of dE2F1 and dE2F2 levels (Dimova et al., 2003). A similar interdependence of interaction partners has also been described for some components of the dMyb complex (Beall et al., 2004).

To demonstrate that Mip130/TWIT is physically bound to E group genes, we performed ChIPs using antibodies specific for dE2F2 and Mip130/TWIT (Figure 5C). Both antibodies precipitated E group promoter sequences (ARPS3D and CG17142) from proliferating S2 cells but failed to enrich a nonspecific control promoter (RP49) (compare lanes 2, 3, and 4). This shows that both dE2F2 and Mip130/TWIT are physically associated with E group genes *in vivo*. Taken together, our results suggest that dREAM complexes assemble on E group genes and effect their permanent repression in proliferating cells.

Consistent with this, levels of dE2F2-regulated gene transcripts were also elevated in embryos and in male and female flies lacking functional Mip130/TWIT in a manner closely resembling *de2f2* mutants (Figure 5D and data not shown) (Dimova et al., 2003). This strongly suggests that dREAM complexes function to repress sex- and differentiation-specific genes in the fly *in vivo*.

dREAM Complexes and synMuv Class B Genes

In *C. elegans*, genes encoding proteins related to RBF (*lin-35*), dE2F (*eff-1*), and dDP (*dpl-1*) are members of the synMuv class B, which, together with synMuv class A and class C genes, antagonize vulval induction (Ceol and Horvitz, 2004). dREAM complexes contain two additional subunits that are related to synMuv class B genes: Mip130/TWIT (*lin-9*) and CAF1p55 (*lin-53*) (Lu and Horvitz, 1998; White-Cooper et al., 2000).

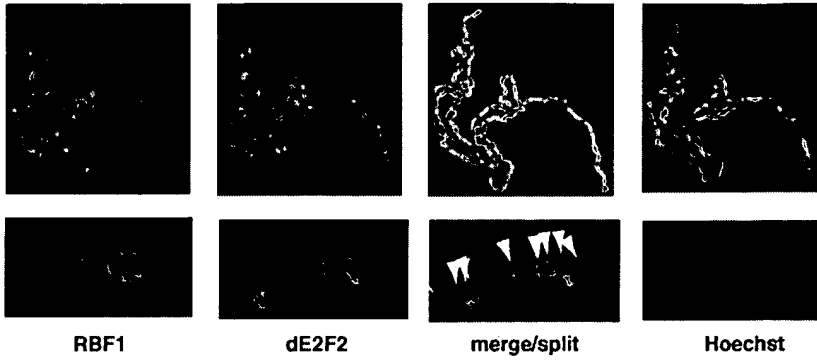
We asked if *C. elegans* homologs of the three remaining dREAM subunits, Mip120, dMyb and Mip40, existed and if they would also antagonize vulval induction. We have been unable to identify a dMyb homolog in *C. elegans* (see Supplemental Data on the Cell web site). In contrast, database searches identified a *C. elegans* gene with extensive similarity to Mip120 (JC8.6; 42% identity and 55% similarity in a region spanning 242 residues). Interestingly, RNAi inactivation of JC8.6 has recently been demonstrated to result in a synMuv phenotype in *lin-8* synMuv class A animals (Owen et al., 2003). We used an RNAi approach to directly compare the penetrance of synMuv phenotypes of three dREAM complex homologs (JC8.6 [p120], *lin-9* [Mip130/TWIT], and *lin-35* [pRb]) in a *lin-15A* synMuv class A background. All three treatments gave rise to multiple pseudovulvae with high penetrance (Figure 6A). JC8.6 RNAi in the synMuv class B background *lin-36(n766)* did not produce synMuv progeny (data not shown). This confirms that the Mip120 homolog JC8.6 has synMuv class B activity *in vivo*.

A BLAST search with the Mip40 sequence revealed several vertebrate homologs but none in *C. elegans*. We therefore aligned *Drosophila* and vertebrate Mip40-related sequences to prepare a profile that was then used to search the database. This identified *C. elegans* Lin-37 as the sequence with the highest similarity to the profile. A sequence related to Lin-37 was also found in the proteome of *C. briggsae*. Alignment of Lin-37- and Mip40-related sequences shows that similarity is confined to three conserved segments (Figure 6B). In each of these segments, there are conserved motifs with features consistent with a globular architecture that is also supported by GlobPlot analysis. We conclude that Lin-37 is a divergent member of the Mip40 family. Intriguingly, *lin-37* is a synMuv class B gene, and a two-hybrid interaction between Lin-37 and Lin-53, the *C. elegans* homolog of the CAF1p55 dREAM subunit, has been reported previously (Walhout et al., 2000).

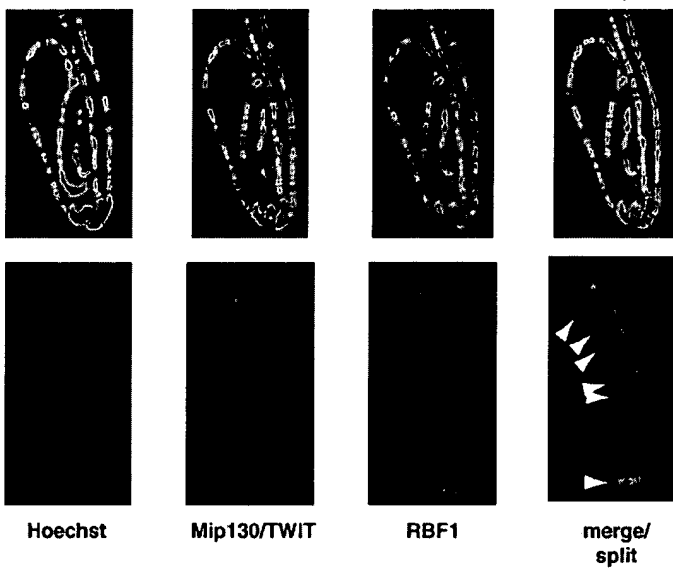
In conclusion, at least seven dREAM subunits are related to synMuv B genes. This raises the possibility that the corresponding gene products function together in a multisubunit complex to regulate vulval cell fate specification in the worm. Our results suggest a remarkable conservation of pRb-related repressor complexes between worm and fly.

The human proteome harbors sequences with signifi-

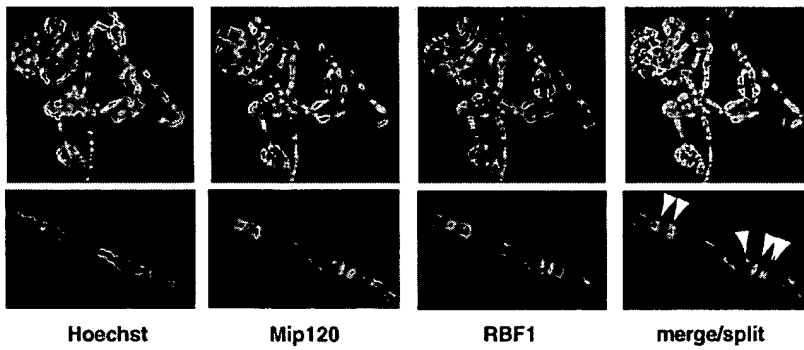
A



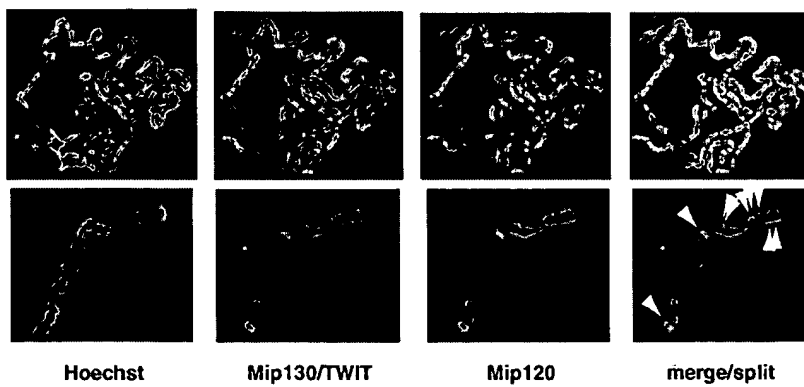
B



C



D



cant similarity to all eight dREAM subunits (data not shown): E2F4/5, DP1/2, RbAp46/48, and B-Myb have all previously been demonstrated to interact with pocket proteins (see Discussion). The putative human homologs of Mip130/TWIT, Mip120, and Mip40 have so far not been characterized. Recombinant hMip130/TWIT, hMip120, and hMip40 all bound specifically to a GST-Rb fusion in vitro (Figure 7A). We have raised an antibody recognizing hMip130/TWIT in Western blots (Figure 7B). The antibody detects two bands in HeLa nuclear extract (Figure 7C): a protein with an apparent molecular weight of 60 kDa, which closely corresponds to the expected molecular weight of hMip130/TWIT (see Supplemental Data); and a smaller protein of 50 kDa, which may represent a degradation product. When GST pull-downs were performed, hMip130/TWIT was strongly retained by GST-pRb fusion protein (compare lanes 1 and 5) but failed to interact with the GST control (lane 4). Binding was diminished by mutation of the LXCXE binding cleft but not eliminated (lane 6). GST-p107 and GST-p130 fusions also bound hMip130/TWIT in this assay (lanes 9 and 10). The smaller antibody-reactive protein failed to bind to any of the GST fusions tested, indicating that the observed association with hMip130/TWIT is specific. Taken together, these results suggest that dREAM subunits and their interaction with pocket proteins have been conserved through evolution.

Discussion

We have purified two native complexes containing dREAM. The two dREAM complexes contain similar subunits but differ with regard to RBF itself: one complex contains RBF1, the other the highly related RBF2. Accordingly, these complexes behave identically during chromatographic fractionation and can only be separated by immunoprecipitation with RBF1- and RBF2-specific antibodies. The properties and identities of dREAM subunits are illuminating, and our results draw together several different research areas: dREAM complexes represent a remarkable point of convergence between studies of E2F-dependent transcription, Myb control of DNA replication, and models of E2F/pRb function in both *Drosophila* and *C. elegans*.

dREAM repressors are required for a recently discovered aspect of dE2F transcriptional regulation. RNAi-mediated disruption of dREAM complexes by depletion of Mip130/TWIT and Mip120 specifically derepresses E group genes (Figure 5), genes we had previously shown to be repressed in a cell cycle-independent manner by dE2F2, dDP, and a function that is redundant between RBF1 and RBF2. Although depletion of Mip130/TWIT and Mip120 had no effect on expression of A group genes, it is probable that dREAM complexes also repress cell cycle-related targets in other situations: ChIP experiments show that dE2F2, RBF1, and RBF2 are nor-

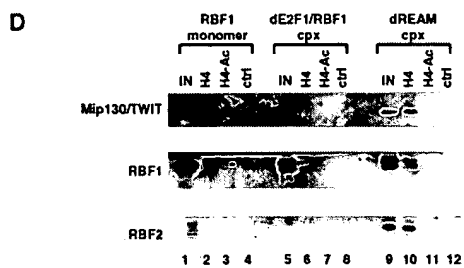
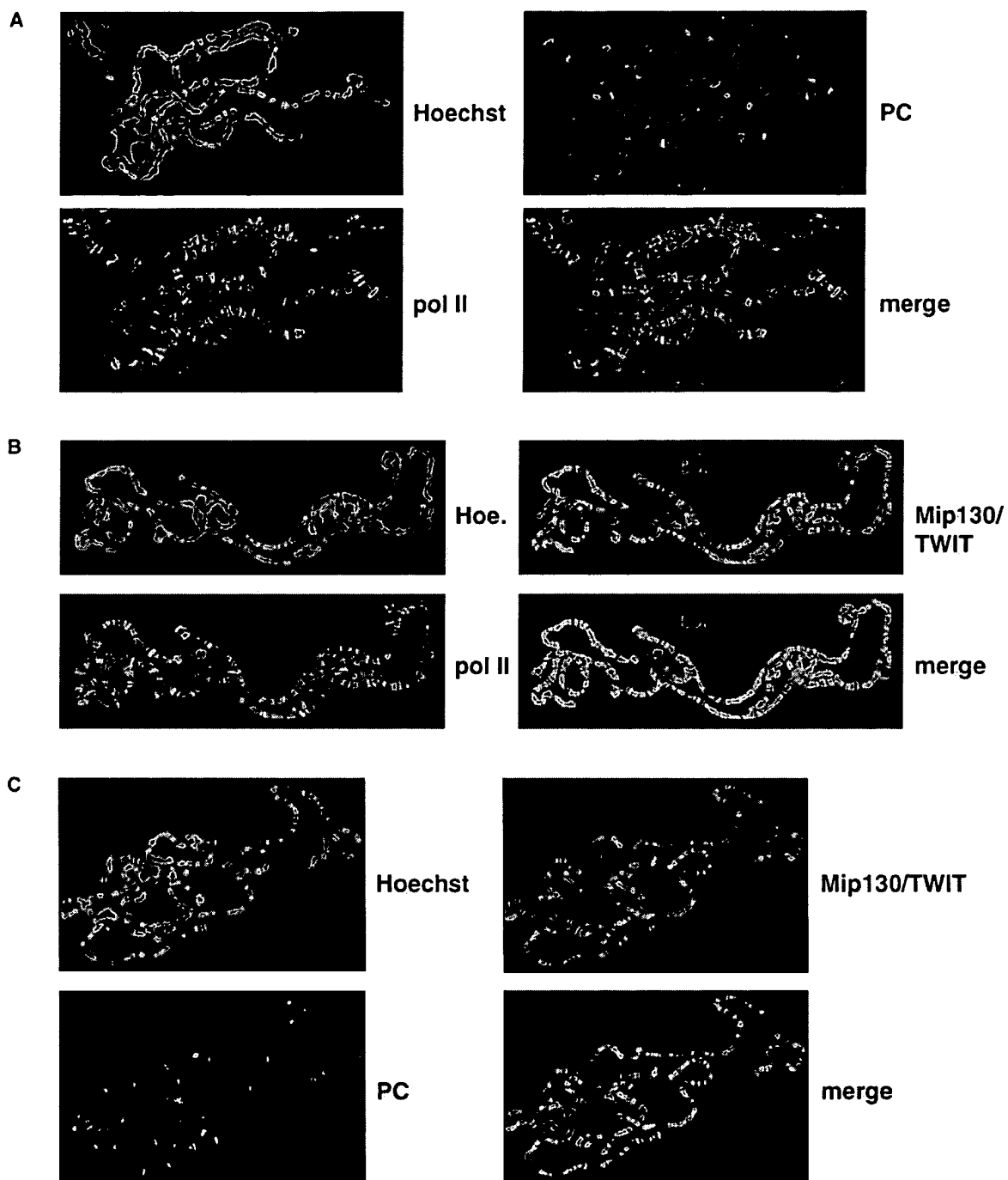
mally present at almost all dE2F-regulated genes, including A group genes; the distinction between A and E group genes lies, therefore, not in the binding of the repressor proteins but in the binding of dE2F1 (Dimova et al., 2003). Accordingly, in cells lacking dE2F1, dE2F2-mediated repression prevents the expression of both cell cycle-dependent and -independent targets (Dimova et al., 2003). The extensive colocalization of dE2F2, RBF1, Mip120, and Mip130/TWIT on polytene chromosomes suggests that dREAM complexes are present at most sites of dE2F action.

The fact that dMyb is a stoichiometric subunit of dREAM complexes hints at an extensive collaboration between dE2F and dMyb. However, depletion of dMyb had no effect on expression of the A and E group genes tested (Supplemental Figure S2). It is clear, therefore, that dMyb is not required for all aspects of dREAM complex function. However, it is possible that dE2F and dMyb cooperate to regulate transcription of other genes that we have not investigated. Moreover, as will be discussed below, dE2F2 and dMyb appear to converge on the regulation of chorion gene amplification.

The mechanism of E2F regulation provided by dREAM appears to be highly conserved during evolution. Strikingly, with the exception of dMyb, all components of dREAM are either homologs of previously described *C. elegans* synMuv class B genes (*mip130/twit/lin-9*, *rbf1* and *rbf2/lin-35*, *de2f2/efl-1*, *ddp/dpl-1*, and *caf1p55/lin-53*), contain regions of sequence conservation (*Mip40/lin-37*), or produce a synMuv phenotype when the corresponding *C. elegans* gene is inactivated (*Mip120/JC8.6*). Genetic studies have shown that synMuv class B genes are required for development of the worm's male and female reproductive systems, and it has been suggested that some encode subunits of a hypothetical complex that represses vulva-specific gene transcription; however, the precise transcriptional changes underlying the synMuv phenotype are unknown (Ceol and Horvitz, 2001). Our discovery of dREAM complexes suggests an intriguing model for synMuv class B gene function: we propose that at least seven synMuv class B gene products physically associate to form a complex that, like its *Drosophila* counterpart, represses sex-related targets and that misexpression of these genes causes a change in cell fate. Given the vast differences between *C. elegans* and *Drosophila* embryogenesis, we consider it unlikely that REAM complexes will regulate the exact same set of genes in both species. However, we propose that, in both organisms, REAM complexes control transcriptional programs required for development of the reproductive system. In agreement with this model, we have previously shown that dE2F2 is needed to repress genes like *vasa* and *spn-E* that are important for *Drosophila* gametogenesis (Dimova et al., 2003) and that dE2F2 mutants have both male and female fertility defects (Cayirlioglu et al., 2001; Frolov et al., 2001).

Figure 3. Localization of dREAM Complex on Polytene Chromosomes

(A) Polytene chromosomes were stained using α -RBF1 (red), α -dE2F2 antibody (green), or Hoechst (blue) as indicated. Bottom panels show a magnified image. Colocalization is visualized by merging (yellow bands, upper panel) and by splitting the magnified image (lower panel). Sites of colocalization are shown by white arrows. (B) Colocalization visualized by α -RBF1 (red) and α -Mip130/TWIT (green), (C) by α -RBF1 (red) and α -Mip120 (green), and (D) by α -Mip120 (red) and α -Mip130/TWIT (green) antibodies.



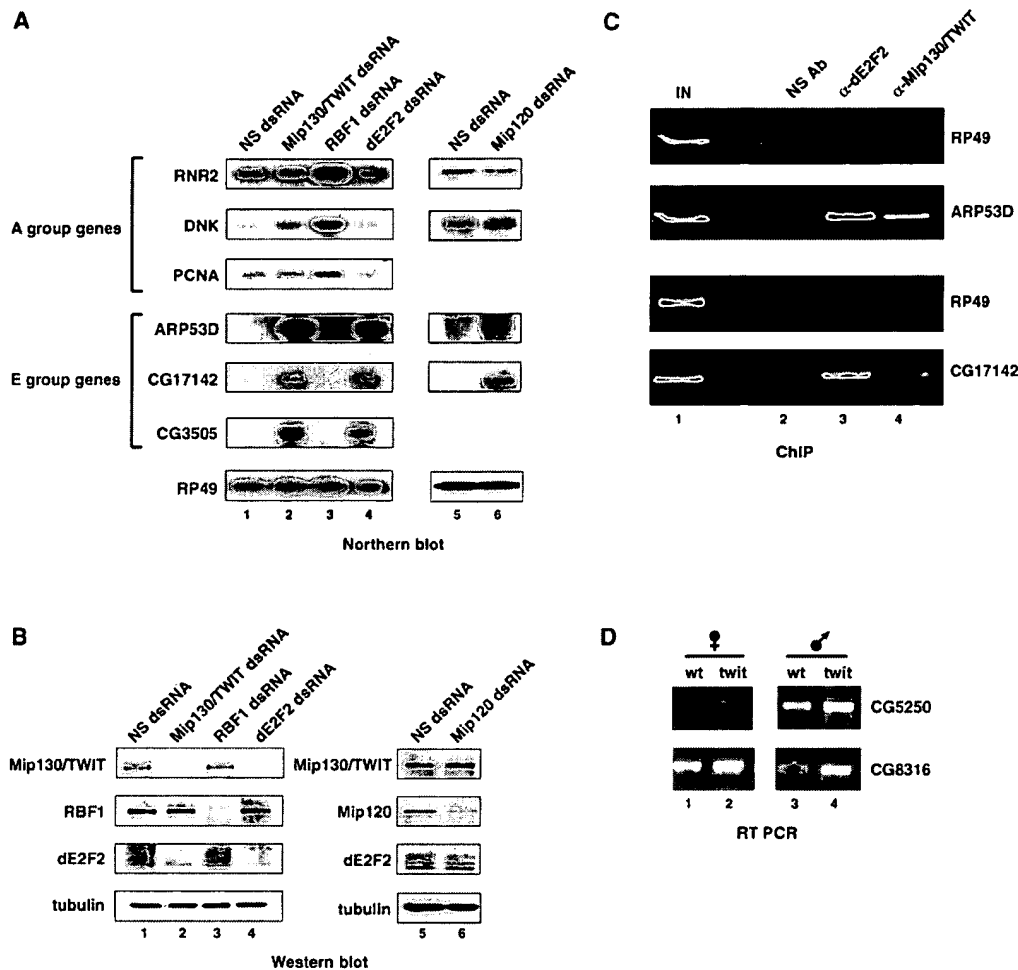


Figure 5. dREAM Complexes Repress E Group Genes

(A) S2 cells were treated with double-stranded RNAs specific for luciferase (NS), Mip130/TWIT, RbF1, dE2F2, and Mip120, as indicated on top. Transcription of three A group genes (*rnr2*, *dnk*, and *pcna*), three E group genes (*arp53D*, *CG17142*, and *CG3505*), and one control gene (*rp49*) was monitored by Northern blot, as indicated on the left.

(B) Extracts from RNAi-treated cells were analyzed by Western blot using specific antibodies, as indicated on the left.

(C) ChIP from S2 cells using α -dE2F2 and α -Mip130/TWIT antibodies, as shown on top. Specific primers were used to amplify enriched promoter sequences, as indicated on the right. IN, genomic DNA input, NS Ab, nonspecific antibody.

(D) Total RNA isolated from wild-type male and female adults (wt) and from *twit* mutant male and female adults was analyzed by RT PCR for the expression levels of dE2F2-regulated genes.

Do mammalian cells contain similar complexes? Mammalian homologs exist for all dREAM subunits. Intriguingly, B-Myb associates with the N terminus of p107 (Joaquin et al., 2002). RbAp48/p46, human orthologs of CAF1p55, were first isolated through their ability to bind a pRb-affinity column (Qian et al., 1993) but are now known as components of several chromatin-associated complexes, including a putative pRb-histone deacetylase and the NuRD complex (Becker and Hörz, 2002; Nicolas et al., 2000). Human homologs of Mip130/TWIT,

Mip120, and Mip40 had not previously been linked to pRb. We find that all three interact with pRb in vitro. Furthermore, endogenous hMip130/TWIT associates specifically with pRb, p107, and p130 fusion proteins. In agreement with our results, Gaubatz and colleagues have recently demonstrated a physical interaction between pRb and Mip130/TWIT in human cells in vivo (S. Gaubatz, personal communication). Clearly, further studies are needed to define the properties and biological roles of pRb/hMip complexes. Nevertheless, our pre-

Figure 4. Mip130/TWIT Binds to Transcriptionally Silent Chromatin

Polytene chromosomes were stained (A) with α -pol II (red) and α -Pc (green), (B) with α -pol II (red) and α -Mip130/TWIT (green), and (C) with α -Pc (red) and α -Mip130/TWIT (green) antibodies. (D) Aliquots of Q Sepharose fractions I (RbF monomer), II (dE2F1/RbF1), and III (dREAM) were incubated with paramagnetic beads loaded with histone H4 peptide (H4), tetraacetylated H4 peptide (H4-Ac), or no peptide (ctrl); extensively washed; and analyzed by Western blot as indicated on the left. IN, input.

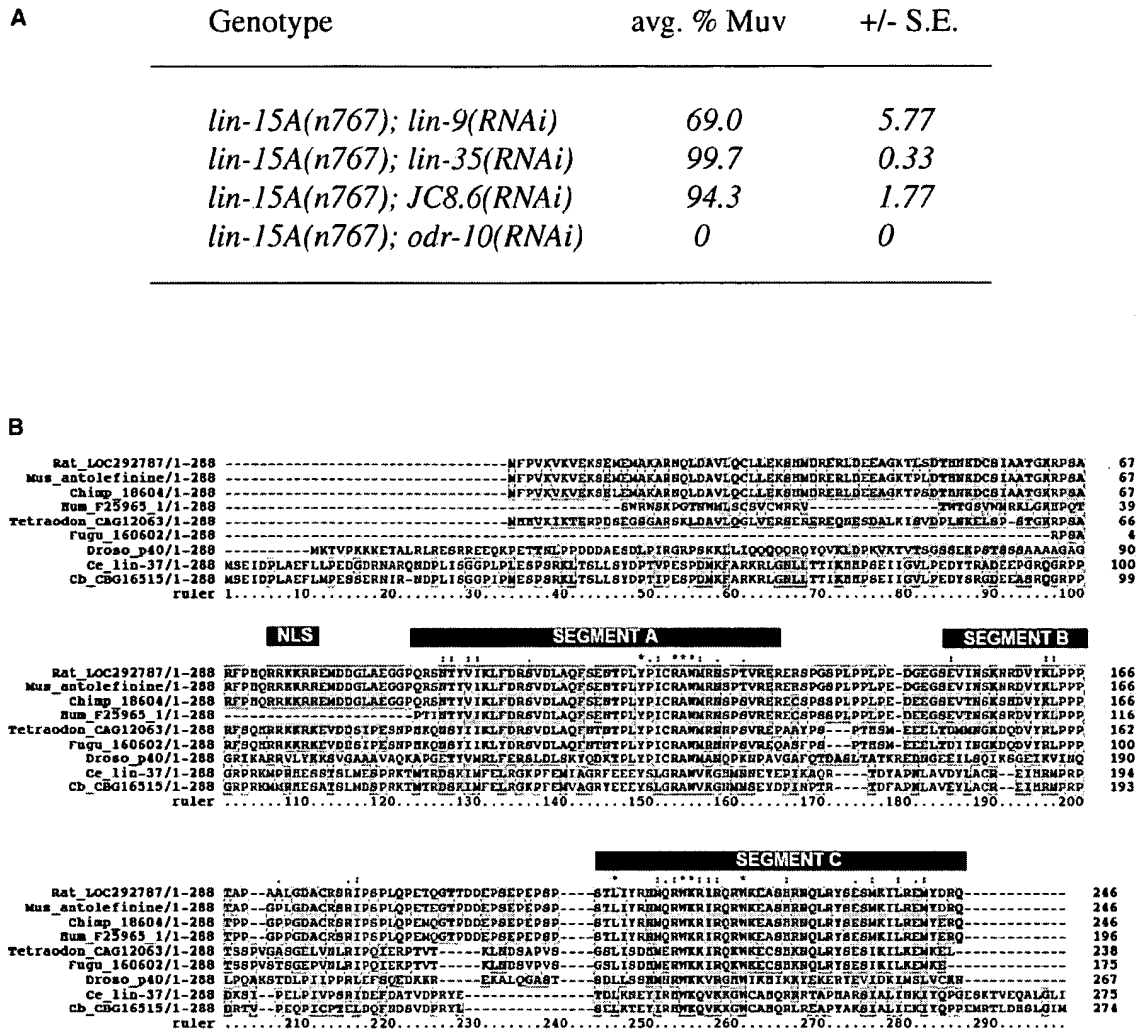


Figure 6. Mip120- and Mip40-Related Genes Antagonize *C. elegans* Vulva Development

(A) *lin-15(n767)* animals were subjected to RNAi depleting *lin-9*, *lin-35*, *JC8.6*, or *odr-10* (control). Average percentage of animals with multiple vulvae from three independent experiments (average % Muv) and standard errors (\pm SEM) are shown (n = 30–115 animals).

(B) Color-coded multiple sequence alignment of Mip40-related and Lin-37 proteins. A putative nuclear localization sequence in the vertebrate sequences (NLS), three segments of similarity (A, B, and C), and a predicted globular region (green line) are shown.

liminary findings suggest that such complexes may well exist in mammalian cells, and, if studies in *C. elegans* and *Drosophila* are a guide, we might expect them to function in developmentally regulated aspects of E2F/PRB function.

What is the biochemical function of dREAM complexes? dREAM complexes lack known chromatin-modifying enzymes. Studies of mammalian E2F targets show that activation and repression correlate with histone acetylation and deacetylation, respectively (Frolov and Dyson, 2004). The finding that dREAM complexes associate specifically with unmodified histone H4 tails but fail to bind hyperacetylated tails implies that they bind specifically to deacetylated histones that are characteristic of repressed chromatin. Consistent with this, dE2F2, RBF, Mip120, and Mip130/TWIT colocalize at chromosomal sites that are not actively transcribed. We propose that dREAM complexes bind deacetylated nucleosomes, protecting them from modification, and in doing so maintain a repressive state that is both stable and readily reversible.

One might predict that dREAM would act synergistically with histone deacetylases. Indeed, the *C. elegans* synMuv B class includes an ortholog of HDAC1, and the dRPD3 histone deacetylase coimmunoprecipitates with RBF from extracts of cell lines (Lu and Horvitz, 1998; Taylor-Harding et al., 2004). However, dRPD3 is not a stoichiometric component of dREAM complexes. Furthermore, inhibition of histone deacetylases in SL2 cells, either by the depletion of dRPD3 or treatment with deacetylase inhibitors, does not derepress group E genes (Taylor-Harding et al., 2004). Thus, while histone deacetylation may be a prerequisite for histone binding by dREAM, deacetylases are not required to maintain repression of E group genes.

The discovery that E2F/RBF complexes contain five

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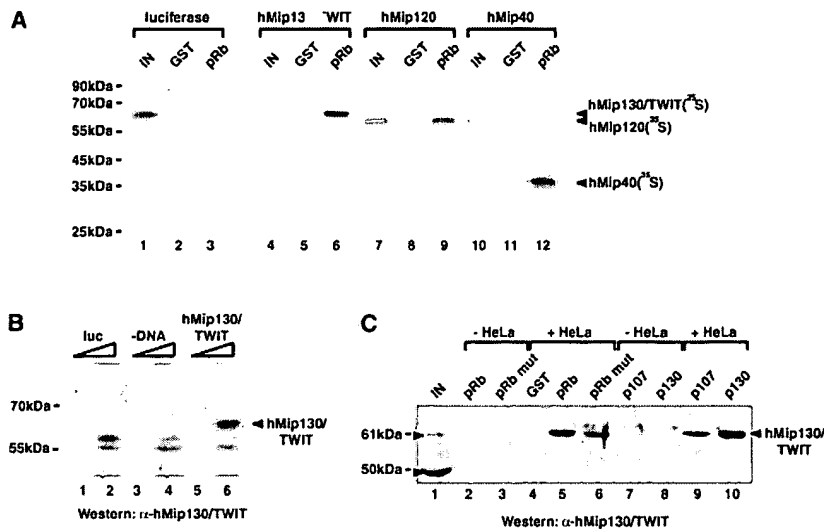


Figure 7. pRb Binds Human Homologs of dREAM Subunits

(A) Immobilized GST (GST) or GST-pRb (pRb) fusions were incubated with ³⁵S-labeled, in vitro-translated luciferase, hMip130/TWIT, hMip120, or hMip40 as indicated. Bound material was subjected to SDS-PAGE and visualized by autoradiography. IN, 5% input.

(B) α-hMip130/TWIT was used to probe in vitro-translated luciferase (lanes 1 and 2) and hMip130/TWIT (lanes 5 and 6). -DNA, no DNA control (lanes 3 and 4).

(C) Immobilized GST fusions were incubated with (+HeLa) or without (-HeLa) HeLa nuclear extract as indicated. Bound material was analyzed by Western blot using α-hMip130/TWIT antibody. The position of antibody-reactive bands is shown by open arrows.

subunits of a recently described dMyb complex is particularly intriguing (Beall et al., 2002, 2004). This complex binds the ACE3 element of a chorion gene locus and has been suggested to regulate chorion gene amplification in ovary follicle cells. Amplification involves both cessation of general genomic replication and relicensing and firing of origins in a temporally and spatially restricted manner (Calvi et al., 1998). Remarkably, dE2F2, dDP, RBF1, and Mip130/TWIT are all needed to shut off genomic replication in vivo (Beall et al., 2004; Bosco et al., 2001; Cayirlioglu et al., 2001; Royzman et al., 1999). Our discovery of dREAM complexes offers a mechanistic explanation for these genetic results and implies that dREAM complexes function to shut off genomic replication in this cell type. Interestingly, dRPD3 and histone deacetylation have very recently been shown to counteract chorion origin firing, lending further support to the idea that "transcriptional" regulators can also influence DNA replication events (Aggarwal and Calvi, 2004).

Recent genetic studies have suggested that the effects of Mip130/TWIT are reversed at rereplicating sequences by dMyb, possibly following an activating modification of dMyb itself (Beall et al., 2004). Interestingly, dE2F1 and dMyb colocalize to amplifying foci, and dE2F1, like dMyb, is needed to promote rereplication (Beall et al., 2002; Bosco et al., 2001; Royzman et al., 1999). Since dE2F1 works at least in part by overriding dE2F2-mediated repression and dMyb has been proposed to selectively counteract Mip130/TWIT activity, the discovery that dE2F2 and Mip130/TWIT reside in the same complex suggests that dE2F1 and activated dMyb may collaborate at the ACE3 locus to reverse repressive effects of dREAM complexes. In this setting, dMyb and E2F appear to share a similar mechanism of action, relying on an activator (dE2F1) or an activating event (modification of dMyb) to relieve the effects of a common repressor.

It should be noted that mutant alleles of *de2f2* and *mip130/twit* are not lethal but do suffer from reduced viability and fertility (Beall et al., 2004; Frolov et al., 2001). Hence, dREAM complexes are not essential. Amplification of chorion loci in follicle cells represents a highly specialized case of DNA replication. The general patterns of DNA replication are unaffected by mutation in *de2f2* and *mip130/twit*, arguing against a strict requirement for replication per se (Beall et al., 2004; Frolov et al., 2001). Nevertheless, several studies of mammalian cells have linked pRb and E2F proteins to various aspects of DNA replication, but their precise roles in replication remain to be established (Angus et al., 2004; Avni et al., 2003; Kennedy et al., 2000; Knudsen et al., 1998).

dREAM complexes are the first native RBF repressor complexes to be purified, but we note that additional complexes likely exist. Our fractionation reveals an additional complex containing RBF1, dE2F1, and dDP (Figure 1B) that might act at other E2F targets that were unaffected by the depletion of dREAM components. Our results show that specific RBF-containing complexes are important at specific subsets of dE2F-regulated promoters. It is becoming clear that pRb/RBF tumor suppressors assemble distinct molecular machines to exert distinct functions. More work is needed to determine which complexes are needed for each of their ascribed functions. The striking parallels between studies of pRb and E2F orthologs in *C. elegans* and *Drosophila* indicate that their basic mechanisms of action are well conserved. Perhaps the most definitive picture will emerge by integrating information from each of the available model organisms.

Experimental Procedures

Purification of dREAM

Nuclear extract from 300 g dechorionated *Drosophila* embryos was successively fractionated over Q Sepharose FF (Amersham), BioRex

70 (Bio-Rad), Q Sepharose HP (Amersham), hydroxylapatite, and Superose 6 (Amersham) columns (see Supplemental Data). Fractions were analyzed by Western blot and silver staining. Bands were excised and analyzed by peptide mass fingerprinting (Zentrum für Proteinanalytik, Adolf-Butenandt-Institut).

Antibodies and Flies

α -hMip130/TWIT was raised in rabbits coinjected with two unrelated peptides derived from the predicted hMip130/TWIT sequence (Eurogentec). Antibodies against *Drosophila* Mip130/TWIT were raised in guinea pigs injected with a C-terminal Mip130/TWIT peptide (Moravian-Biotechnology, details available on request). Mip130/TWIT, Mip120, dMyb, Mip40 (M. Botchan), and Pc (R. Paro) antibodies were generous gifts. Immunoprecipitations and Western blots were carried out using standard procedures (Bouazoune et al., 2002).

The generation and characterization of *Mip130/twit* mutant flies will be presented elsewhere (H.W.-C., unpublished data).

Polytene Staining

Polytene stainings were done following standard protocols (Sullivan et al., 2000). A mixture of two monoclonal RBF1 antibodies was used at dilutions of 1:4/1:2; α -dE2F2, α -Mip120, and α -Pc (rabbit) at 1:100; α -Mip130/TWIT (rabbit) and α -Twt 2.1 (guinea pig) at 1:250; and α -pol II (mouse; H5 [Covance]) at 1:150. As secondary antibodies, Cy3-conjugated goat anti-mouse or anti-rabbit antibodies (1:400) and Cy2-conjugated goat anti-rabbit or anti-guinea pig antibodies (1:300) were used (Jackson Laboratory). DNA was counterstained with Hoechst 33258 (Sigma). Polytene chromosomes were analyzed using a computer-controlled Zeiss Axiophot microscope equipped with a cooled CCD camera (QIMAGING).

Peptide Binding Assays

Q Sepharose HP fractions containing monomeric RBF1, dE2F1/RBF1, or dREAM were tested for binding to unmodified and modified histone tail peptides. Amounts of RBF1 in each fraction were adjusted by Western blot. Fractions were diluted with binding buffer (20 mM Tris [pH 8.0], 100 mM KCl, 10% glycerol, 0.2% NP-40, and PMSF) to a final volume of 500 μ l. Peptide pulldown assays were carried out essentially as described (Bouazoune et al., 2002).

Cell Culture, RNAi, ChIP, RT-PCR, and Northern Blot

SL2 cells were cultured at 25°C in Schneider's insect medium (Sigma; 10% fetal bovine serum). SL2 cell lines stably expressing Flag-tagged RBF1 and RBF2 were established and maintained as described (Bouazoune et al., 2002). RNA interference, ChIP, RT-PCR, and Northern blots were performed as in Dimova et al. (2003), Frolov et al. (2001), and Stevaux et al. (2002).

C. elegans Feeding RNAi

MT1806 *lin-15(n767)* and MT6034 *lin-36(n766)* strains were maintained on modified growth medium containing 0.2% lactose to induce dsRNA expression (Eric Lambie, personal communication). Plates were seeded with *E. coli* harboring RNAi feeding constructs and grown for several hours at 37°C. About ten bleached L1 animals were added and raised at 25°C. Progeny that possessed more than one vulva-like structure were scored as Muv. RNAi feeding constructs were from the Ahringer and Vidal libraries. Details are available on request.

Sequence Analysis

For details on database searches and accession numbers, see Supplemental Data.

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